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Antiviral Inhibition of the HIV-1 Capsid Protein

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During the assembly stage of the human immunodeficiency virus (HIV) replication cycle, several thousand copies of the viral Gag polyprotein associate at the cell membrane and bud to form an immature, non-infectious virion. Gag is subsequently cleaved by the protease, which liberates the capsid proteins for assembly into the polyprotein shell of the central core particle (or capsid) of the mature virus. Viral infectivity is critically dependent on capsid formation and stability, making the capsid protein a potentially attractive antiviral target. We have identified compounds that bind to an apical site on the N-terminal domain of the HIV-1 capsid protein and inhibit capsid assembly *in vitro*. One compound, N-(3-chloro-4-methylphenyl)-N'-[2-[(15-[(dimethylamino)-methyl]-2-furyl)-methyl]-sulfanyl]ethylurea (CAP-1), is well tolerated in cell cultures, enabling *in vivo* antiviral and mechanistic studies. CAP-1 inhibits HIV-1 infectivity in a dose-dependent manner, but does not interfere with viral entry, reverse transcription, integration, proteolytic processing, or virus production, indicating a novel antiviral mechanism. Significantly, virus particles generated in the presence of CAP-1 exhibit heterogeneous sizes and abnormal core morphologies, consistent with inhibited CA-CA interactions during virus assembly and maturation. These findings lay the groundwork for the development of assembly inhibitors as a new class of therapeutic agents for the treatment of AIDS.

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Introduction

Currently available drugs for the treatment of human immunodeficiency virus (HIV) infection target the reverse transcriptase (RT) and protease

Abbreviations used: CA, capsid protein (p24); CAP-1, N-(3-chloro-4-methylphenyl)-N'-[2-[(15-[(dimethylamino)-methyl]-2-furyl)-methyl]-sulfanyl]ethylurea; CAP-2, 1-(4-(N-methylacetamido)-phenyl)-2-(4-methyl-3-nitrophenyl)urea; CTD, carboxy-terminal domain; CV, cell viability; Gag, precursor polyprotein (p55); Gag²⁸³, 283-residue N-terminal fragment of the HIV-1 Gag polyprotein; HAART, highly active anti-retroviral therapy; HBV, hepatitis B virus; $\Delta\delta$, chemical shift change induced upon ligand binding; HIV-1, human immunodeficiency virus type-1; HSQC, heteronuclear single quantum coherence; IU, infectious units; K_d , dissociation equilibrium constant; MA, matrix protein (p17); NMR, nuclear magnetic resonance; NTD, amino-terminal domain; PR, protease; RT, reverse transcriptase.

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(PR) enzymes, two of fifteen proteins encoded by the viral genome. These drugs are marginally effective when administered independently due to the rapid emergence of resistant strains that are selected under conditions of incomplete viral suppression.¹ Although sustained reductions in viral load can be achieved when inhibitors are used in appropriate combinations (highly effective anti-retroviral therapy, HAART),^{1,2} inadequate suppression due to poor compliance, resistance, and interactions with other drugs or diet is a significant problem that limits the effectiveness of HAART therapy for many patients and can lead to the spread of drug-resistant strains.^{1–5} Inhibition of other viral or cellular components may provide the best approach for attacking viral resistance.¹ One potential target that has yet to be exploited is the capsid protein (CA). CA is initially synthesized as a domain within a 55 kDa Gag precursor polyprotein. Approximately 4,000 copies of Gag assemble at the plasma membrane and bud to form an immature virus particle.⁶ Subsequent to

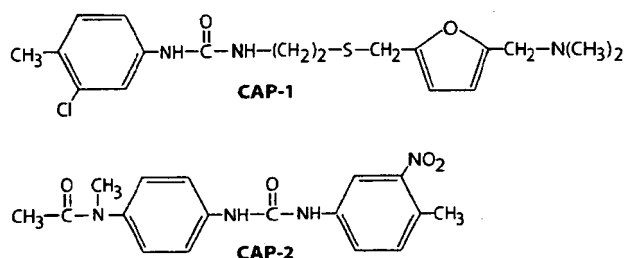


Figure 1. Chemical structures of the HIV-1 capsid assembly inhibitors CAP-1 and CAP-2.

budding, CA is liberated by proteolytic cleavage of Gag, which triggers a conformational change that promotes assembly of the capsid particle.⁷⁻¹⁰ Two copies of the viral genome and enzymes essential for infectivity become encapsidated in the central, cone shaped capsid of the mature virion.

Several recent studies have shown that proper capsid assembly is critical for viral infectivity.^{8,9,11-13} Mutations in CA that inhibit assembly are

lethal,^{8,9,11,12} and mutations that alter capsid stability severely attenuate replication,¹³ making the capsid protein an attractive potential antiviral target. Antiviral agents have been developed that bind to the capsid proteins of picornaviruses (including the human coxsackie, rhino- and polioviruses) and suppress infectivity by inhibiting disassembly of the capsid shell,¹⁴ and inhibitors of hepatitis B virus (HBV) capsid assembly have recently been identified.¹⁵ Although similar strategies were anticipated for HIV more than a decade ago,¹⁶ inhibitors of capsid assembly or disassembly have yet to be identified, possibly due to the significant structural differences between the retroviral (predominantly helical) and non-retroviral (predominantly β -sheet) proteins.^{7,17,18}

Results and Discussion

Identification of a ligand binding site on the N-terminal domain of CA

In an attempt to identify agents that inhibit functions of the capsid protein, we computationally

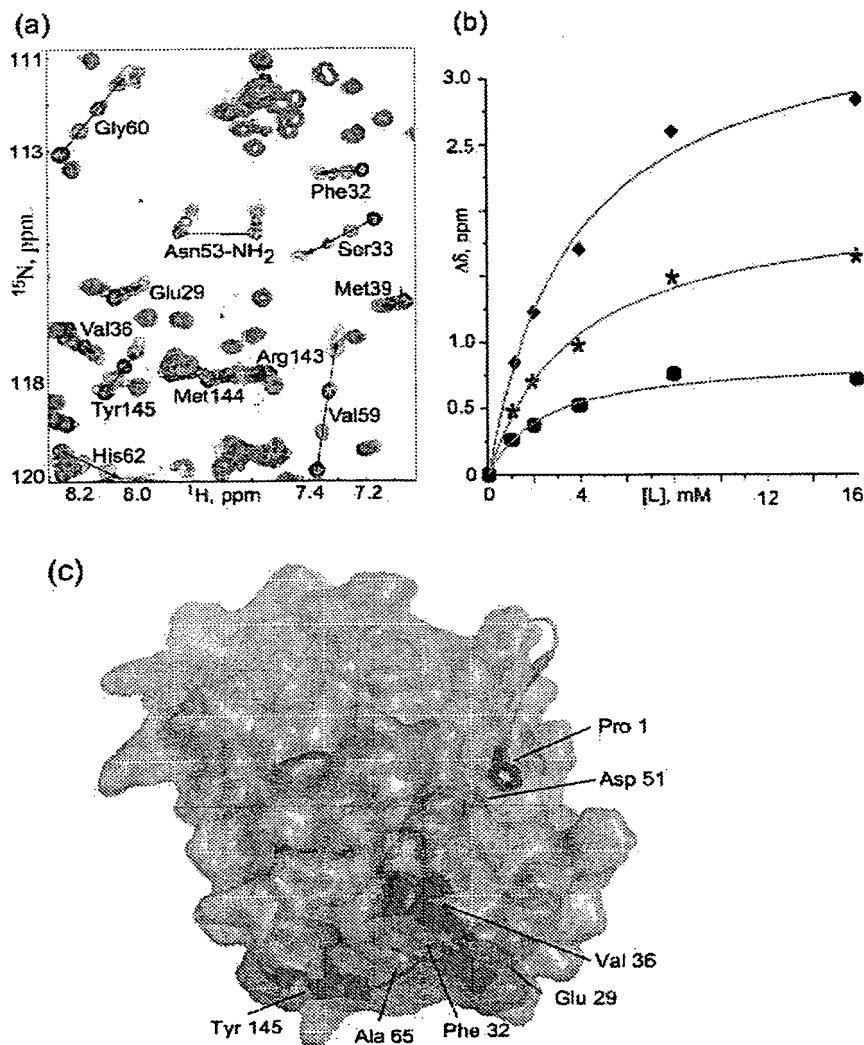


Figure 2. (A) Overlay of 2D ^1H - ^{15}N HSQC spectra obtained for the HIV-1 capsid protein N-terminal domain (CA NTD) upon titration with CAP-1. Arrows denote progressive shifts with increasing [CAP-1]. Colors and CAP-1:CA ratios are as follows: 1:0, black; 1:1, red; 1:4, blue; 1:8, green; 1:16, magenta. (B) Representative ^{15}N NMR chemical shift titration data for residues Ser 33 (squares), Val 59 (diamonds) and Gly 60 (stars) and fit to 1:1 binding isotherms ($K_d = 0.82 \pm 0.18$ mM). (C) Structure of the mature HIV-1 CA NTD showing the N-terminal β -hairpin (Pro1 in blue and Asp51 in red). Residues with backbone amide ^1H and ^{15}N signals that shift by more than 0.1 and 0.5 ppm, respectively, upon saturation with CAP-1 are colored green (image generated with pymol⁴³).

screened public-domain chemical libraries for compounds that might bind to clefts on the surface of the capsid protein and tested for binding using nuclear magnetic resonance (NMR) titration spectroscopy. Screening efforts focused mainly on a " β -hairpin cleft" that is exposed on the surface of the N-terminal domain (NTD) of the immature capsid protein but becomes occupied by residues of a β -hairpin that forms after proteolytic cleavage of Gag.¹⁰ Compounds from public domain chemical libraries were screened using DOCK-4.0,¹⁹ and 40 compounds with good theoretical binding properties (binding energy < -26 kcal/mol, Contact Score < -40 ; further details to be reported elsewhere) were experimentally tested for binding to the intact CA protein, the NTD of CA, and a 283 residue fragment of the immature Gag polypeptide (Gag²⁸³).¹⁰ Although we have yet to identify agents that bind to the β -hairpin cleft, this process led to the fortuitous discovery of compounds that bind to a different site that also appears to be important for capsid assembly.

Representative ^1H - ^{15}N HSQC NMR data obtained upon titration of the mature NTD with N-(3-chloro-4-methylphenyl)-N'-[2-[[[5-[(dimethylamino)-methyl]-2-furyl]-methyl]-sulfanyl]-ethyl]-urea (CAP-1, Figure 1) are shown in Figure 2A. Although most signals were unaffected by the titrations, a subset of signals shifted as a function of increasing CAP-1 concentration, indicating site-specific binding. The chemical shift changes fit to 1:1 binding isotherms and afforded an equilibrium dissociation constant (K_d) of 0.82 ± 0.18 mM at 35 °C, Figure 2B. Significantly tighter binding was observed for a second compound, 1-(4-(N-methylacetamido)phenyl)-3-(4-methyl-3-nitrophenyl)urea (CAP-2, Figure 1; $K_d = 52 \pm 27$ μM). In both cases, the CA residues perturbed by binding ($^1\text{H}_\text{N}$ $\Delta\delta > 0.1$ ppm; ^{15}N $\Delta\delta > 0.5$ ppm; Glu29, Lys30, Ala31, Phe32, Ser33, Glu35, Val36, Val59, Gly60, Gly61, His62, Gln63, Ala65, Met144 and Tyr145) are located at or near the apex of a helical bundle (helices 1, 2, 3, 4 and 7), Figure 2C. Essentially identical results were obtained for titrations with Gag²⁸³ and intact CA, indicating that the binding site remains accessible in Gag-like constructs containing the native N-terminal matrix (MA) and C-terminal capsid (CTD) domains, and that binding is insensitive to the maturation state of the protein.

Inhibition of capsid assembly *in vitro*

In the absence of other viral constituents, HIV-1 CA can assemble into tubes with structural features that resemble mature cores.^{8,20-22} Tube formation leads to increases in sample turbidity that can be monitored spectrophotometrically,²³ and this assay was used to probe for potential inhibitory effects of the CAP compounds on *in vitro* capsid assembly. As shown in Figure 3, dissolution of native HIV-1 CA into assembly buffer (50 mM phosphate buffer, pH 8.0, 2.5 M NaCl, 0.04% v/v DMSO) led to an increase in absorbance at an

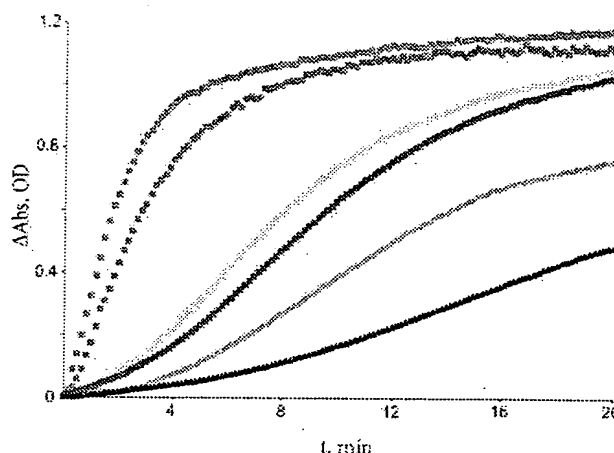


Figure 3. Turbidity assay results showing the effects of CA-binding compounds on *in vitro* capsid assembly. Compound: CA ratios and initial rates (millioptical density units (mOD) per minute, reported as the mean \pm standard deviation from three experiments) are as follows: red, 0.2 μL DMSO control (204 ± 36 mOD/min); magenta, 1:1 bis-(2-thiomethylbenzimidazole)methane (does not bind CA) (309 ± 4 mOD/min); yellow, 1:1 CAP-1 (93 ± 3 mOD/min); brown, 2:1 CAP-1 (67 ± 16 mOD/min); green, 0.5:1 CAP-2 (81 ± 2 mOD/min); blue, 1:1 CAP-2 (39 ± 11 mOD/min).

initial rate of 204 ± 36 mOD/min (determined from the initial slope and reported as the mean \pm standard deviation from three experiments). As expected, compounds tested that do not bind CA did not affect the rate of assembly. However, the assembly rate was diminished in a dose-dependent manner by both CAP-1 and CAP-2, with the more tightly binding CAP-2 having a more pronounced effect. As shown in Figure 3, the initial assembly rates in the presence of CAP-1 decreased to 93 ± 3 and 67 ± 16 mOD/min at CAP-1: CA ratios of 1:1 and 2:1, respectively. For comparison, assembly rates in the presence of the more tightly binding CAP-2 decreased to 81 ± 2 and 39 ± 11 mOD/min at CAP-2: CA ratios of 0.5:1 and 1:1, respectively. These data confirm that CA-binding compounds can inhibit capsid assembly *in vitro*, and that the relative efficacy of assembly inhibition is dependent on the affinity of the ligands for the CA protein.

Inhibition of viral infectivity

The CAP compounds were tested for toxicity and antiviral activity using HIV-1 producing, latent infected U1 cells. This assay allows assessment of antiviral effects on late phase replication events.²⁴ Although CAP-2 was too cytotoxic for *in vivo* evaluations, CAP-1 was non-toxic under the conditions employed, and its application led to dose-dependent reductions in supernatant infectivity, Figure 4A. At 100 μM CAP-1, the U1 cells were fully viable, but infectivity was reduced by ca. 95% relative to untreated samples, Figure 4A,B. To

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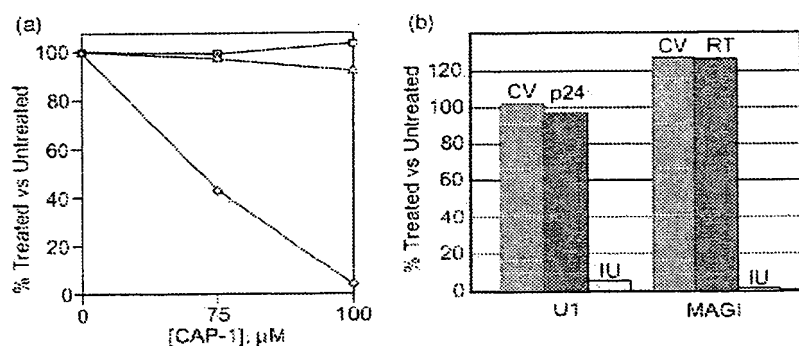


Figure 4. (A) Viral infectivity (diamonds), cell viability (CV, squares) and virus production (p24 levels, triangles) from latent infected U1 cultures as a function of added CAP-1. CAP-1 does not inhibit cell growth or virus production, but the particles produced are poorly infectious. (B) Cell viability (CV), virus particle associated reverse transcriptase activity (RT) and p24 (CA) levels, and infectious units (IU) obtained upon incubation of infected U1 and MAGI cells for 72 hrs with 100 μM CAP-1. Values are reported as percentages relative to untreated cells.

determine if CAP-1 affects viral gene expression and particle production, reverse transcriptase (RT) activity and CA (p24) levels were measured for the supernatants after pelleting and removal of the cells. As shown in Figure 4B, both the CA levels and RT activities were unaffected by CAP-1, indicating that antiviral activity is not due to inhibited virus production. In addition, the p24 (CA) levels observed in Western data obtained for treated and untreated samples were very similar, Figure 5, indicating that CAP-1 does not significantly affect proteolytic processing of Gag. Consistent with this finding, CAP-1 did not affect *in vitro* protease activity. The p24 Western data also indicate a reduction in the intracellular levels of Gag (p55) as a function of increasing levels of CAP-1, whereas the levels of the Gag cleavage products p24, p41 remained relatively unaffected. Although further studies are warranted, these findings suggest that CAP-1 promotes the intracellular degradation of the full-length Gag polypeptide. Quantitation of gp120 was also obtained for the supernatant using antibodies against gp120. No

differences were observed between the treated and untreated samples, indicating that CAP-1 does not inhibit the synthesis or viral incorporation of the envelope glycoprotein (data not shown).

Antiviral activity was also tested in a second cellular assay using MAGI cell cultures. As observed in the U1 assay, treatment of infected, virus producing MAGI cells with CAP-1 led to dose-dependent reductions in virus particle infectivity, with infectious units dropping by nearly two log units to less than 2% of the untreated levels at 100 μM CAP-1, Figure 4B. No reductions in viral RT activity were observed, providing further evidence that antiviral activity is not due to inhibited virus production. Virus production was also unaffected by pre-incubation of either MAGI cells or virus particles with CAP-1, indicating that CAP-1 is not virucidal and does not directly inhibit early phase events. These data collectively indicate that the antiviral activity of CAP-1 is due to the inhibition of a late-phase viral event that is different from events targeted by other anti-HIV agents currently under investigation or in clinical use.

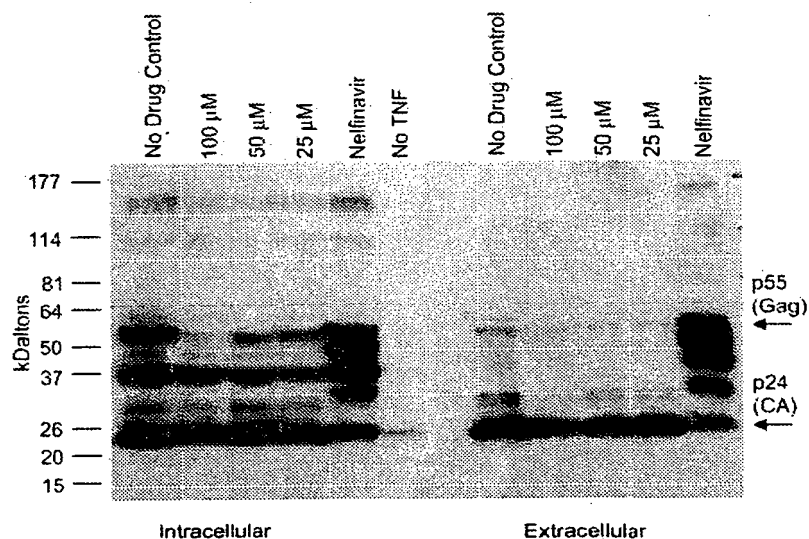


Figure 5. Capsid protein (p24) Western blot assays showing relative concentrations of intra- and extracellular (viral) CA in the free (24 kDa band), Gag polyprotein (55 kDa band) and partially processed states as a function of added CAP-1. As controls, results obtained upon incubation with the protease inhibitor Nelfinavir (at low dose, for observation of proteolytic intermediates), and without addition of tumor necrosis factor (TNF, required for induction) are also shown.

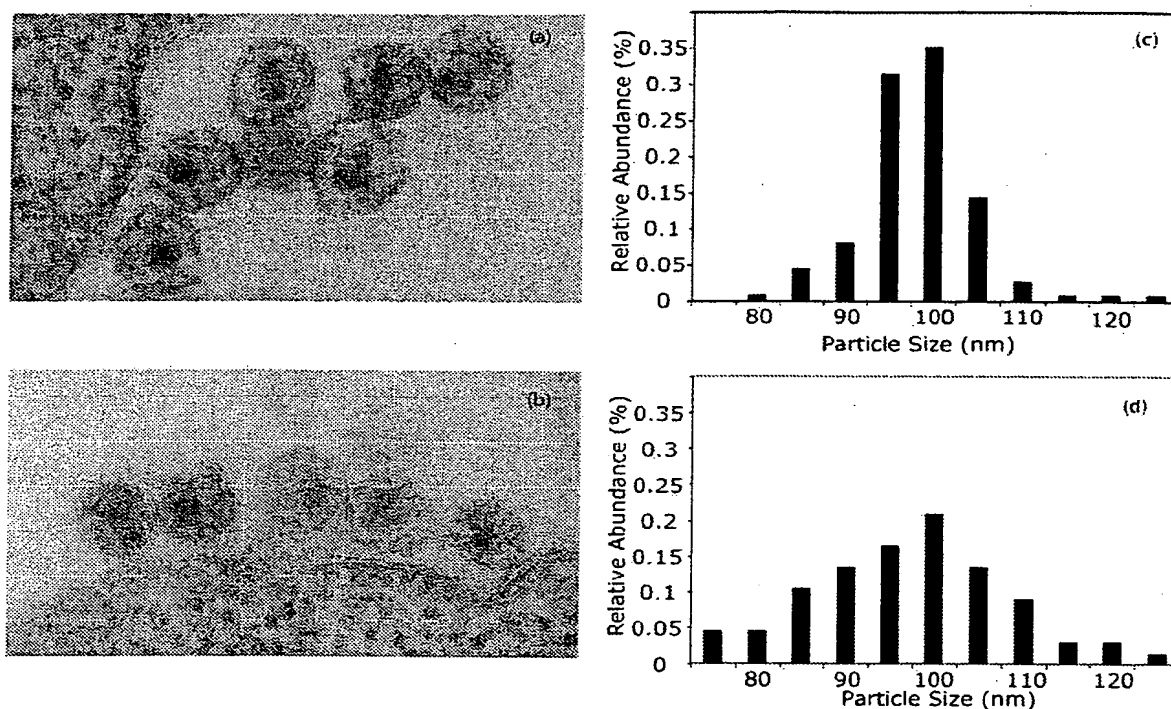


Figure 6. EM images and size distributions of virus particles generated in the absence (A and C, respectively) and presence (B and D, respectively) of 100 μ M CAP-1. Treatment results in the production of a more heterogeneous population of virions consistent with inhibited or altered Gag–Gag interactions during virus assembly. Treated particles also exhibit aberrant core morphologies, which indicates that CAP-1 disrupts CA–CA interactions and inhibits capsid assembly during maturation.

Inhibition of Capsid Assembly *in vivo*

Since CAP-1 did not inhibit virus production, it was possible to examine the virions produced from treated cells for morphological defects by electron microscopy (EM). The capsids of mature virions generally appear as central, conical (~30% of the particles) or spherical (~40%) structures, depending on the orientation of the cone during thin section sample preparation, and about 30% of virions in EM preparations typically exhibit an immature phenotype characterized by the lack of central electron density.^{8,12,25–27} We obtained similar results for virions generated from untreated U1 cell cultures. However, as shown in Figure 6, particles generated from the treated cells exhibited greater size heterogeneity, which is indicative of a Gag assembly defect. In addition, only 35% of the particles from the treated cells contained a dense, centrally located core, compared to 70% from the untreated cells. Most strikingly, none of the particles from the treated cells exhibited a cone-shaped core that is the hallmark of HIV, indicating that CAP-1 inhibits assembly of the mature core. Essentially identical phenotypes were observed previously for virions generated with CA mutations designed to disrupt intermolecular CA–CA interactions.^{8,12,27,28} Thus, the EM data indicate that CAP-1 inhibits capsid assembly during viral maturation, and interferes to some extent with normal Gag–Gag interactions during assembly of the immature particle.

Antiviral Mechanism and Implications for AIDS Chemotherapy

The CAP compounds identified here bind to a common site on the N-terminal domain of CA in both its mature and immature forms. Residues of CA with backbone amide signals that are most significantly perturbed by binding are either strictly conserved (Glu35, Val36, Val59, Gly60, His62, Gln63, Ala65 and Tyr145) or rarely and conservatively substituted (number of occurrences in parentheses: E29D (2) K30R (1), A31G (16), A31N (1), F32L (1), S33N (13), G61E (1), M144T (1)) among the 93 genome sequences in the HIV Sequence Compendium.²⁹ Most of the conserved residues are exposed on the surface of the NTD, suggesting a possible macromolecular interactive function. Although high resolution structural information for an assembled HIV-1 capsid is currently unavailable, very recent mass spectrometric studies indicate that several of the conserved residues of the apical site participate in a CA–CA interface upon *in vitro* capsid assembly.³⁰ In these studies, backbone amide hydrogens of apical site surface residues that exchanged readily with solvent (D_2O) deuterons became protected from exchange when the protein assembled into tubes.³⁰ In addition, Lys 71 of the NTD, which is adjacent to the apical site, was shown to be susceptible to chemical cross-linking with a lysine on the CTD of a second capsid molecule during mild alkylation of the

tubes. These results collectively indicate that residues of the apical site of the NTD participate in an intermolecular CA(NTD)-CA(CTD) interface upon *in vitro* capsid formation.³⁰ In combination with our present findings, these data provide compelling evidence that the CAP compounds function mechanistically by inhibiting intermolecular CA-CA interactions necessary for proper capsid assembly.

Residues Trp23 and Val59 exhibit significant chemical shift changes upon CAP ligand binding, despite the fact that they are buried between helices 1, 2 and 3 of the CA monomer. It is therefore likely that the assembly inhibitors alter the local structure of the capsid protein, and may thereby either competitively inhibit CA-CA interactions or promote the formation of a structurally distorted capsid shell. Binding-induced distortions would also explain why the computational screening procedure did not identify the apical site for high affinity ligand binding, since the protocol employed does not allow for protein flexibility.^{19,31,32} Binding-induced protein conformational changes are not uncommon and have confounded computational approaches to structure-based drug design.³² For example, non-nucleoside inhibitors of HIV-1 reverse transcriptase interact within a binding-induced cavity that is not present in the native, unliganded protein.³³

Mutagenesis studies from several laboratories have shown that HIV-1 infectivity is highly sensitive to mutations in CA and to the stability of the capsid.^{8,9,11-13} Particles with aberrant capsid morphologies are non-infectious,¹² and those with cores that are normal in appearance but have altered stability (both elevated and reduced, due to mutations in CA) exhibit severely attenuated infectivity.¹³ As such, agents that marginally affect capsid assembly or stability have the potential to be potent inhibitors of viral replication. Our studies indicate that inhibition of capsid assembly does not require ligands with exceptionally high affinity for CA. This is likely due to the high local concentration of Gag molecules in assembled virions (~14 mM), which favors binding by ligands with even modest affinities such as CAP-1. Thus, conservatively assuming that cytosolic drug concentrations in the budding virus and cells are equal (100 μ M), the percentage of viral CAP-1 molecules bound to CA can be estimated by standard mass action calculations (viral diameter $D = 96$ nm; intraviral volume $= \pi D^3/6 = 463,250$ nm³; 4,000 Gag molecules/virion; $[Gag] = 14$ mM; $[CAP-1] = 100$ μ M; $K_d = 820$ μ M $= ([CA] - [CA:CAP-1])([CAP-1] - [CA:CAP-1])/[CA:CAP-1]$, which affords a value for the concentration of bound CAP-1 ($[CA:CAP-1]$) of 94 μ M. This indicates that 94% of the CAP-1 molecules in immature virions (100 μ M dose) should be bound to Gag, and that binding to as few as ~25 molecules of Gag per virion are sufficient to inhibit core assembly during viral maturation.

The Western data presented here also suggest that CAP-1 promotes the intracellular degradation of the Gag precursor protein. Gag normally functions by recruiting cellular factors associated with the vacuolar protein sorting and degradation machinery for assistance in budding,³⁴⁻³⁷ and it is plausible that the binding of CAP-1 to the capsid domain of Gag could alter this process and lead to the targeting of greater quantities of Gag for proteasomal degradation.

The present results provide proof of principle for the development of antiviral inhibitors of HIV-1 capsid assembly. Since compounds with higher affinity for cytosolic Gag will be concentrated in assembling viruses, and compounds with greater affinity for CA are more potent inhibitors of *in vitro* assembly, it should be possible to rationally design agents with increased potency.

Materials and Methods

Sample Preparation and NMR Spectroscopy

DNA encoding the CA NTD (residues 1 through 151) was amplified from HIV-1 cDNA plasmid pNL-4-3³⁸ and an oligonucleotide encoding a C-terminal hexa-histidine tag was appended to the gene. The DNA was inserted into a p11a expression vector (Novagen, Madison, WI), and the protein product was purified by cobalt affinity chromatography (Clontech, Palo Alto, CA); $MW_{calc} = 17523.0$ daltons, $MW_{obs} = 17523.10 \pm 0.44$ daltons (electrospray Mass Spectrometry). The plasmid for the full-length, native capsid protein was kindly provided by Dr. W. I. Sundquist (Utah), and the protein was purified as described.²³ NMR spectra were assigned using conventional triple resonance methods.³⁹ Binding isotherms from ¹H-¹⁵N NMR HSQC titration experiments were calculated with ORIGIN 7.0 software (MicroCal, Northampton, MA). CAP-1 was from Maybridge (HTS 02911; Cornwall, England) and CAP-2 and bis-(2-thiomethylbenzimidazole)methane were from Sigma-Aldrich (S85,367-4 and RCL S4,007-0, respectively; Milwaukee, WI).

In Vitro Assembly

Turbidity assays were performed at 21 °C using a Beckman DU650 spectrophotometer operating at 350 nm wavelength. Concentrated ligand in DMSO (0.2 μ l) was added to a 250 μ l aqueous solution containing the capsid protein ($[CA] = 60$ μ M; $[NaH_2PO_4] = 50$ mM; pH 8.0). Particulates were removed by centrifugation, and capsid assembly was initiated by addition of a concentrated NaCl solution (5 M, 250 μ l). Spectral measurements were made every 10 s, following a short initial delay to allow sample equilibration. Relative assembly rates were estimated from initial slopes of the plots of absorbance versus time.

In Vivo Infectivity

U1 cells (5×10^5 cells/ml) were mixed with TNF- α (10 ng/ml, Sigma) for activation of HIV virion production and treated with CAP-1 at different concentrations. Cultures were harvested 72 hrs after treatment.

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Cell viability was measured using the MTS cell proliferation assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI). Supernatants were collected, the cell debris removed by low speed centrifugation, and the particles in the supernatants pelleted by microcentrifugation. Infectious units associated with the particles were measured as described previously⁴⁰ except that β -Gal activities were measured using a Tropix Gal-Screen detector system (Applied Biosystems, Foster City, CA). Particle-associated RT activities were determined as described.⁴¹ Cell lysates and pelleted particles were subjected to SDS-PAGE analysis as described previously⁴² using AIDS patient sera (AIDS Research and Reference Regent Program, NIAID, NIH). Quantitative p24 (CA) assays were performed with the HIV-1 p24 Antigen Capture ELISA kit (AIDS vaccine program, FCRDC/SAIC/NCI, Frederick, MD). Quantitative gp120 assays were performed with the HIV-1 gp120 Antigen Capture ELISA Kit (Advanced Biotechnologies Inc., Columbia, MD). MAGI cells were washed after viral adsorption (HIV-1_{RF}) with PBS and were fed fresh media containing CAP-1 at various concentrations. Seventy-two hours post infection, the culture supernatants were harvested and pre-cleared. Virus particles present on the supernatants were collected by microcentrifugation and particle-associated RT activity and infectivity were subsequently measured.

Electron Microscopy

Treated (CAP-1) and untreated virus-producing U1 cells were pelleted, washed in phosphate-buffered saline (PBS), and resuspended in at least ten cell pellet volumes of fixative (100 mM sodium cacodylate, pH 7.2, 2.5% glutaraldehyde, 1.6% paraformaldehyde, 0.5% picric acid). Cells were fixed for 24–48 h, after which fixative was removed, and cells were washed twice in PBS, and then pelleted in eppendorf centrifuge tubes. Washed cell pellets were post-fixed 1 h in 1% osmium tetroxide plus 0.8% potassium ferricyanide in 100 mM sodium cacodylate, pH 7.2. After thorough rinsing in water, cells were pre-stained in 4% uranyl acetate 1 h, thoroughly rinsed, dehydrated, infiltrated overnight in 1:1 acetone:Epon 812, infiltrated 1 h with 100% Epon 812 resin, and embedded in the resin. After polymerization, 60–80 nm thin sections were cut on a Reichert ultramicrotome, stained 5 min in lead citrate, rinsed, post-stained 30 min in uranyl acetate, rinsed and dried. EM was performed at 60 kV on a Philips CM120/Biotwin equipped with a 1024 × 1024 Gatan multiscan CCD, and images were collected at original magnifications of 26,880 × –36,960 ×, corresponding to resolutions of 8.9 and 6.5 Å/pixel, respectively. For each sample, 4 separate EM grids were viewed, and at least 47 images were collected, corresponding to a minimum total area of 35 micron².

Acknowledgements

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